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CELLULAR UPTAKE AND METABOLISM OF DAUNORUBICIN AS DETERMINED BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

APPLICATION TO L1210 CELLS

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SUMMARY

The metabolism of daunorubicin (DNR) to daunorubicinol and daunomycinone in murine leukaemia cells has been examined by means of high-performance liquid chromatography. A rapid and efficient extraction method has been developed that permits the recovery of the drug and its metabolites from cell homogenates. By means of high-performance liquid chromatography daunorubicinol and daunomycinone have been separated from DNR and the intracellular concentration of the compounds determined. The method developed is very rapid and sensitive, and amounts as small as 30 pg of DNR can be detected. The results indicate that the aldo-keto reductase is not very active in L1210 cells in culture, the main intracellular product found being DNR.

INTRODUCTION

Since daunorubicin (DNR) is used as an antitumour agent in the treatment of acute leukaemias and malignant lymphomas, the quantitative determination of the drug and its metabolites in tissues and cells is becoming more important both clinically and experimentally. Previous methods required a purification step and were time consuming. Langone *et al.*[1] recently suggested the use of high-performance liquid chromatography (HPLC) for the determination of doxorubicin (DOX, 14-dihydrodaunorubicin) and its metabolites in urine and Hulhoven and Desager[2] used HPLC to determine DNR and its main metabolite daunorubicinol (DOL, 13-dihydro-DNR) in plasma.

We describe here a method for the extraction of DNR and its metabolites from cell homogenates and their quantitative determination by HPLC down to a concentration of 30 pg (1.5 ng/ml).

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MATERIALS AND METHODS

DNR, DOL and DOX hydrochloride were generously supplied by Dr. Maral (Rhône-Poulenc, Paris, France). The solvents were of analytical grade (Merck, Darmstadt, G.F.R.) and were used without purification. Phosphate-buffered saline (PBS) at pH 7.4 has the following composition: NaCl, 140 m*M*; KCl, 3 m*M*; KH_2PO_4, 8 m*M*; Na_2HPO_4 8 m*M* in deionized water.

Drug-uptake experiments

L1210 leukaemia cells were cultivated in suspension[3] using RPMI 1640 medium (Eurobio, Paris, France), supplemented by 10% fetal calf serum[4]. $3 \cdot 10^5$ L1210 cells were incubated at 37° for 24 h in 2 ml of medium before addition of DNR at a concentration of 1 μg/ml. After various times the cells were centrifuged and washed with PBS.

Extraction procedure

Cells were suspended in 2 ml of PBS and sonicated at 50 W for 20 sec with a Branson B-12 sonicator (Branson, Conn., U.S.A.). A 0.1-ml sample of the internal standard (DOX at 10 μg/ml) and 1.8 ml of the extraction mixture (chloroform–methanol 4:1) were then added to 0.1 ml of the cell homogenate. The sonication tubes were stoppered with silicone elastomer corks and the contents sonicated again for 15 sec. 20 μl of the lower organic layer were then injected directly into the chromatograph using a 100-μl Hamilton syringe.

High-performance liquid chromatograph

A Hewlett-Packard Model 1084 high-performance liquid chromatograph was used with a six-port injection valve (Rheodyne, Model 7120 with a loop of 20 μl). The stationary phase consisted of 7-μm silica-gel particles prepacked into a 250 × 3 mm stainless-steel column (Hibar, LiChrosorb Si 60; Merck). The mobile phase was chloroform–methanol–glacial acetic acid–water (720:210:35:30) at a flow-rate of 1.0 ml/min.

A Gilson Fl-IA/B fluorimeter (Gilson, Middleton, Wisc., U.S.A.) was connected in series with the Hewlett-Packard detector (254 nm wavelength). Excitation and emission wavelengths of 480 nm and 560 nm were selected by use of the corresponding interference filters. The detection and integration of the peaks were performed by the built-in Model 1080 software integrator of the chromatograph. Drug concentrations were calculated by taking into account their specific fluorescence (see Fig. 1).

RESULTS AND DISCUSSION

A limit of sensitivity of 10 ng/ml DNR could be obtained by using the built-in UV detector at maximal amplification (10^{-4} absorbance units per cm). An increased selectivity and also a greater sensitivity were obtained by using the Model F1-IA/B fluorimeter connected in series with the Hewlett-Packard detector. The lowest amount of DNR detectable is 1.5 ng/ml. This amount is defined by the height of the DNR peak being three times that of the noise level.

Calibration graphs for an equimolar mixture of DNR, DOL and DOX are

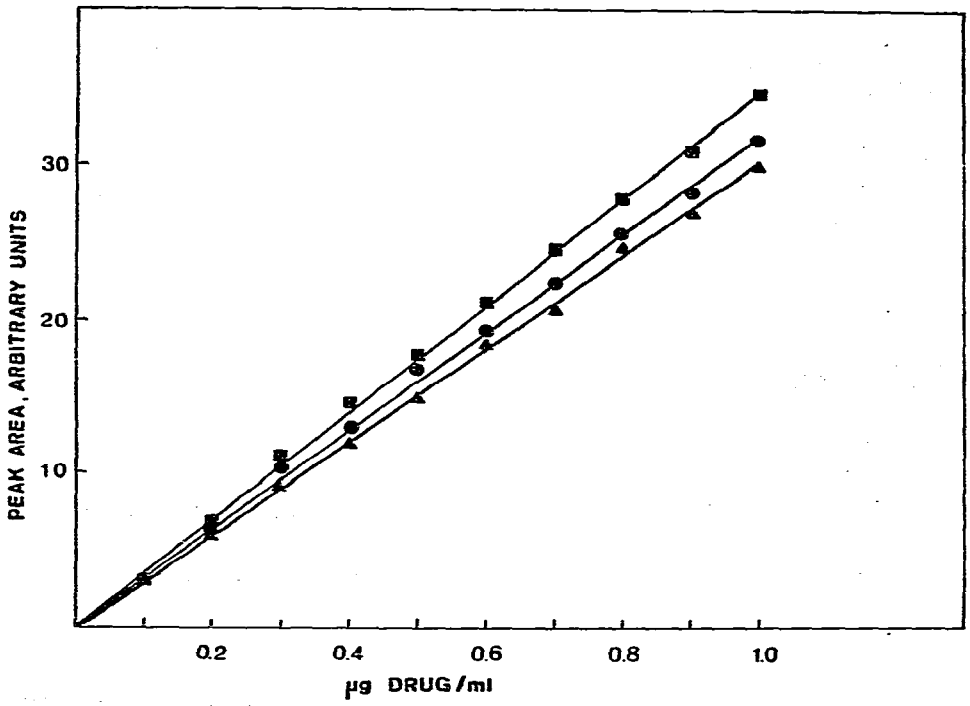

Fig. 1. Calibration curves of DNR (■), DOL (●) and DOX (▲) as measured by HPLC. The integrated peak areas given were obtained from the integrator of the fluorimeter. The flow-rate was set at 1 ml/min.

rectilinear in the range 10–1000 ng/ml (Fig. 1). The reproducibility of the retention times and the accuracy of the integrated surfaces are excellent, as shown in Table I.

The elution system of chloroform–methanol–acetic acid–water was chosen in order to separate DNR from its metabolites in less than 10 min. Fig. 2 shows the separation of four compounds related to DNR. It can be seen that the peaks are sharp and well resolved. From the ratio of retention time (t_R) and baseline bandwidth (w)

TABLE I

REPRODUCIBILITY OF THE RETENTION TIMES AND PEAK AREAS OBTAINED BY HPLC OF DNR, DOL AND DOX IN AN EQUIMOLAR MIXTURE (0.18 n*M*/ml)

Twelve different samples of the equimolar mixture were injected into the liquid chromatograph. The flow-rate was set at 3.0 ml/min. The retention times and areas given for each run by the 1080 software integrator were averaged and the standard deviations calculated.

Sample constituents	*Retention time (min)*		*Peak area (integrated counts × 10^3)*	
DNR	2.38	± 0.01	25.36	± 0.72
DOL	3.03	± 0.01	24.97	± 0.98
DOX	3.30	± 0.02	21.64	± 0.95

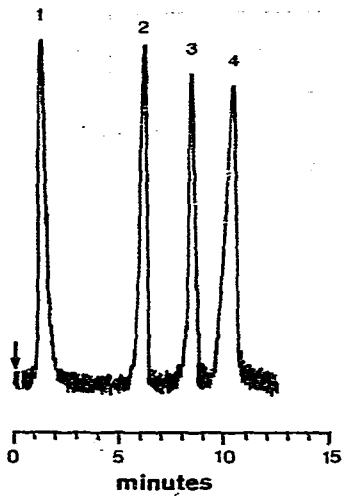

Fig. 2. Chromatogram of a sample mixture of daunomycinone (1), DNR (2), DOL (3) and DOX (4).

we have calculated, using eqn. 1 (ref. 5), an efficiency of 3100 theoretical plates (N) for our 25-cm column.

$$N = 16(t_R/w)^2 \quad (1)$$

The retention times for the compounds reflected their hydrophilic character since the most apolar compound daunomycinone (the aglycone of DNR), is eluted first (t_R = 1.4 min) while the more polar hydroxyl derivatives DOL (t_R = 8.7 min) and DOX (t_R = 10.1 min) are eluted after DNR (t_R = 6.4 min).

We have used this method to determine the uptake and metabolism of DNR by leukaemia L1210 cells grown in culture. As shown in Fig. 3, the recovery of DNR

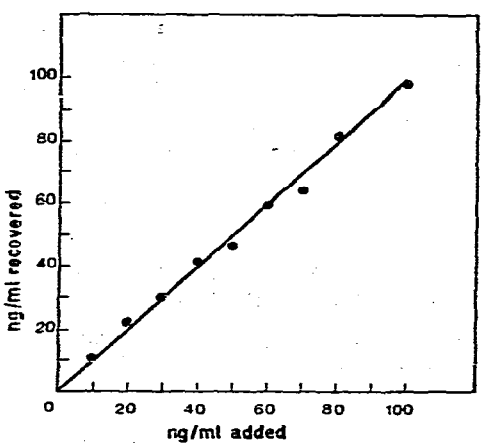

Fig. 3. Recovery of DNR from an L1210 cell homogenate. DNR was added to $3 \cdot 10^5$ L1210 cells in 1 ml of RPMI medium in order to obtain drug concentrations of 10–100 ng/ml. Each tube was sonicated for 20 sec at 50 W. To 0.1 ml of L1210 cell homogenate was added 0.1 ml of DOX at 10 μg/ml borate buffer at pH 9.8 and the drugs were extracted with 1.8 ml of chloroform–methanol (4:1). 20 μl of the organic phase were injected into the chromatograph.

from L1210 cell extracts is close to 100%. The same extraction procedure gave complete recovery of DNR from mouse whole blood[6]. The time course of the uptake and metabolism of DNR by L1210 cells, as determined by our methods, is illustrated in Fig. 4. The rate of uptake of DNR is linear up to 150 min and the accumulation reaches a plateau level of 3.0 μg/mg of cell protein after 5 h. This result indicates that DNR is accumulated about 600 times by L1210 cells if we assume by comparison with cultivated fibroblasts[7] that 1 mg of cell protein corresponds to a volume of 5 μl. The transformation of DNR into DOL proceeds very slowly, only 3% of DNR being metabolized after 22 h. Significant amounts (up to 10% of the total fluorescence) of daunomycinone were also detected after 22 h of incubation of the cells in presence of the drug.

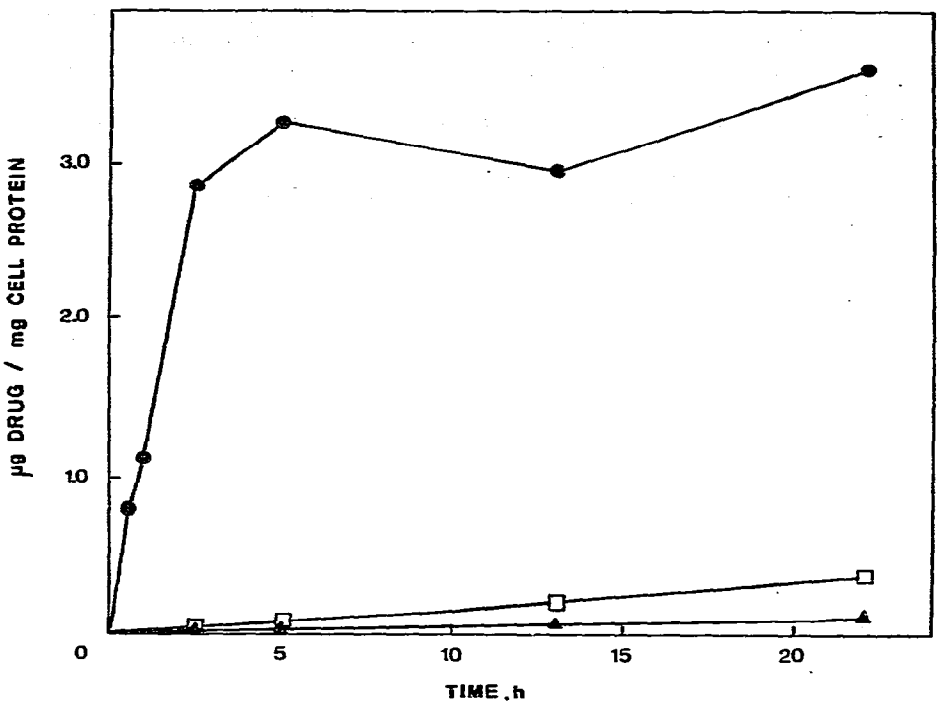

Fig. 4. Intracellular uptake and metabolism by L1210 leukaemia cells *in vitro*. $3 \cdot 10^5$ L1210 cells in 2 ml of RPMI medium supplemented with 10% calf serum were incubated in the presence of DNR at 1 μg/ml. Samples were taken at intervals and to 0.1 ml of cell homogenate were added 0.1 ml of DOX (internal standard) at 10 μg/ml of borate buffer (pH 9.8), and 1.8 ml of chloroform–methanol (80:20). After sonication, 20 μl of the lower organic layer were injected into the chromatograph. ●, DNR; □, daunorubicin aglycone; ▲, DOL.

The method described allows a rapid determination of DNR and its fluorescent metabolites in cells since it takes, at the most, 15 min to obtain the chromatogram subsequent to harvesting of the cells. The sensitivity is very high since it is possible to detect drug concentrations down to 1.5 ng/ml. DNR is easily distinguished from its main metabolites since the chromatogram peaks are sharp and well resolved.

However, our method does not allow the separation of very apolar derivatives, like the agiycones of DNR described by Takanashi and Bachur[8]. Moreover, polar anthracyclines like DOX interact strongly with the silica gel, thus precluding the use of our method for rapid separation of DOX and its polar derivatives.

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